PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

James E. GALEN

Appln. No.: 09/993,292

Confirmation No.: 5386

Filed: November 23, 2001

For: USE OF ClyA HEMOLYSIN FOR EXCRETION OF PROTEINS

Docket No: A8461

Group Art Unit: 1645

Examiner: Duffy, P.

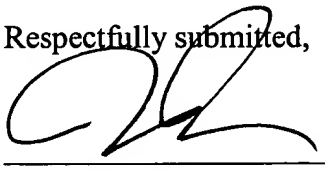
SUBMISSION OF EXECUTED DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith is a copy of an executed Declaration Under 37 C.F.R. §1.132 signed by James E. GALEN.

Respectfully submitted,



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For: USE OF ClyA HEMOLYSIN FOR EXCRETION OF PROTEINS

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, James E. Galen, hereby declare and state:

THAT I am a citizen of the United States of America;

THAT I have received the degree of Ph.D. in 1991 from the University of Maryland, Baltimore;

THAT I have been employed by the Center for Vaccine Development since 1993, where I hold a position as Associate Professor, with responsibility for engineering expression systems for attenuated *Salmonella enterica* serovar Typhi human live vector vaccine strains.

U.S. patent application 09/993,292 discloses my work pertaining to the development of a bacterially-derived protein export system for efficiently producing recombinant protein in a bacterial host cell. The method is generally based on linking a polynucleotide encoding a bacterial export protein to a polynucleotide encoding a protein of interest. The polynucleotide encoding this fusion protein is inserted into a bacterial expression vector. Host cells are then transfected with the expression vector and cultured under conditions promoting production of the

fusion protein. Fusion protein exported from the host cells is then collected from the culture medium.

The bacterial export proteins that I used in my method are those of the HlyE family of export proteins. The prototypic member of the HlyE family is the HlyE protein expressed by *E. coli*. As described in paragraph 0022 of the pending application, *E. coli* HlyE is a 303 amino acid protein that forms stable, transmembrane pores in lipid bilayers. Other members of the HlyE family include *Salmonella enterica* serovar Typhi (*S.* Typhi) cytolysin A (ClyA) protein, *Salmonella paratyphi* ClyA protein, and *Shigella flexneri* hemolysin E (HlyE) protein.

As shown in Appendix I filed with the instant Declaration, the *E. coli* HlyE protein, the *Salmonella enterica* serovar Typhi (*S.* Typhi) cytolysin A (ClyA) protein and the *Salmonella paratyphi* ClyA protein, each recited in the claims of the pending application, are highly homologous. Indeed, a comparison of these three proteins reveals 272 out of 305 identical amino acids over the entire length of these proteins. Thus, it is clear that these three proteins are very highly homologous.

Experiments conducted by Wallace et al. (*Cell* 100:265-276 (2000)) and Atkins et al. (*J. Biol. Chem.* 275:41150-41155 (2000); both filed concurrently herewith) demonstrated that the *E. coli* HlyE protein could be mutated in such a manner that the hemolytic activity of the protein was attenuated. The mutations were the following mutations: G180V, V185S, A187S, and I193S. A recent experiment I performed in collaboration with Jeff Green, one of the authors of the Wallace et al. publication, have demonstrated that the triple mutant identified by Wallace et al. (V185S, A187S, and I193S) is exported from *E. coli* (results shown Appendix III). Thus, in

addition to attenuation of hemolytic activity, the mutated *E. coli* HlyE protein retains its function as an export protein.

As the Cly proteins of *S.* Typhi and *S. paratyphi* have a conserved amino acid at position 185 (isoleucine) and the same amino acids at positions 187 and 193, mutations in these three positions in the Cly proteins of *S.* Typhi and *S. paratyphi* would be expected to have the same result, namely, attenuation of hemolytic activity and maintenance of the export function.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/04/04

James E. Galen

APPENDIX I

CLUSTAL W (1.82) multiple sequence alignment

```
S.typhiClyA          MTSIFAEQTVEVVKSAIETADGALDLYNKYLDQVIPWKTFDETIKELSRFKQEYSQEASV 60
S.paratyphiClyA      MTGIFAEQTVEVVKSAIETADGALDFYNKYLDQVIPWKTFDETIKELSRFKQEYSQEASV 60
E.coliHlyE           MTEIVADKTVEVVKNAIETADGALDLYNKYLDQVIPWQTFDETIKELSRFKQEYSQAASV 60
                     ** *.*::******.**********:***********:****************** ***

S.typhiClyA          LVGDIKVLLMDSQDKYFEATQTVYEWCGVVTQLLSAYILLFDEYNEKKASAQKDILIRIL 120
S.paratyphiClyA      LVGDIKVLLMDSQDKYFEATQTVYEWCGVVTQLLSAYILLFDEYNEKKASAQKDILIRIL 120
E.coliHlyE           LVGDIKTLLMDSQDKYFEATQTVYEWCGVATQLLAAYILLFDEYNEKKASAQKDILIKVL 120
                     ******.**********************.****:**********************::*

S.typhiClyA          DDGVKKLNEAQKSLLTSSQSFNNASGKLLALDSQLTNDFSEKSSYFQSQVDRIRKEAYAG 180
S.paratyphiClyA      DDGVNKLNEAQKSLLGSSQSFNNASGKLLALDSQLTNDFSEKSSYFQSQVDRIRKEAYAG 180
E.coliHlyE           DDGITKLNEAQKSLLVSSQSFNNASGKLLALDSQLTNDFSEKSSYFQSQVDKIRKEAYAG 180
                     ***:.********** ************************************:********

S.typhiClyA          AAAGIVAGPFGLIISYSIAAGVIEGKLIPELNNRLKTVQNFFTSLSATVKQANKDIDAAK 240
S.paratyphiClyA      AAAGIVAGPFGLIISYSIAAGVIEGKLIPELNDRLKAVQNFFTSLSVTVKQANKDIDAAK 240
E.coliHlyE           AAAGVVAGPFGLIISYSIAAGVVEGKLIPELKNKLKSVQNFFTTLSNTVKQANKDIDAAK 240
                     ****:*****************:********:::**:******:** *************

S.typhiClyA          LKLATEIAAIGEIKTETETTRFYVDYDDLMLSLLKGAAKKMINTCNEYQQRHGKKTLFEV 300
S.paratyphiClyA      LKLATEIAAIGEIKTETETTRFYVDYDDLMLSLLKGAAKKMINTCNEYQQRHGKKTLLEV 300
E.coliHlyE           LKLTTEIAAIGEIKTETETTRFYVDYDDLMLSLLKEAAKKMINTCNEYQKRHGKKTLFEV 300
                     ***:******************************** *************:*******:**

S.typhiClyA          PDVAS 305
S.paratyphiClyA      PDI-- 303
E.coliHlyE           PEV-- 303
                     *::
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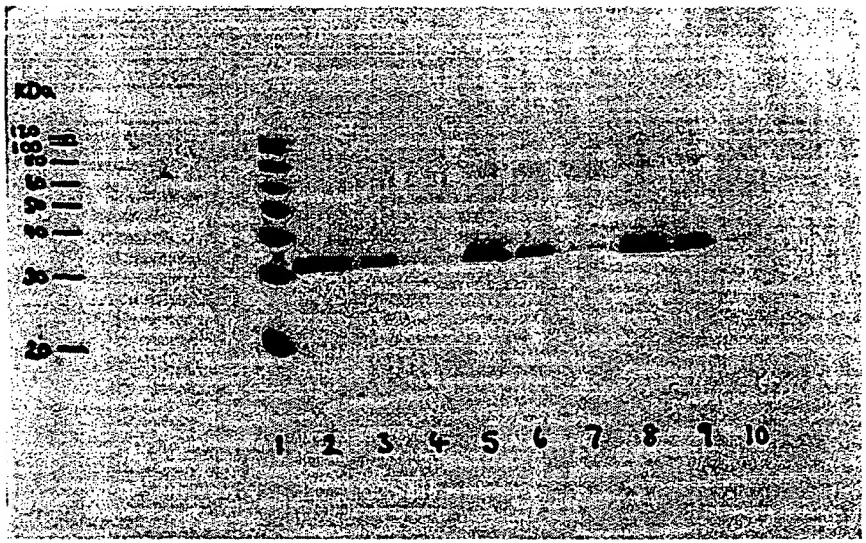
APPENDIX II

Hi Jim

The results of the blots on wild-type HlyE, the triple mutant and the R188 variant are in. Tiff file attached.

Key to track loadings (all ppt'd supernat. protein samples):

1. MW markers - sizes shown on Fig.
2. WT
3. Triple mutant (V185S/A187S/I193S)
4. Double mutant (A187G/G188R)
5. WT
6. Triple
7. Double
8. WT
9. Triple
10. Double



Expt.al Details:

HlyE over-expressed (1mM IPTG, 2-3h) in triplicate aerobic cult.s. Supernatants harvested at cult. OD 600 = 1.0 - 1.1. Supernat.s conc'd ~100-fold (evaporation/drying). Proteins ppt'd (MeOH/Chloroform/dH20) and re-dissolved in Urea/SDS buffer.

Approximately equal quantities of protein loaded per track of SDS- PAGE gel and Western blotted. ECL-Plus development after probing with anti-HlyE primary- and HRP-conjugated secondary antibodies.
Looks like the triple mutant is exported ok.